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Sensitive and Specific Detection of *Pseudomonas avellanae* using Primers based on 16S rRNA Gene Sequences

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With 4 figures

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Abstract

A rapid polymerase chain reaction (PCR)-based procedure was developed for the detection of *Pseudomonas avellanae*, the causal agent of hazelnut (*Corylus avellana*) decline in northern Greece and central Italy. The partial sequence of the 16S rRNA gene of *P. avellanae* strain PD 2390, isolated in central Italy, was compared with the sequence coding for the same gene of *P. syringae* pv. *syringae* type-strain LMG 1247_{t1}. Primers PAV 1 and PAV 22 were chosen, and after the PCR, an amplification product of 762 base pairs was specifically produced only by 40 strains of *P. avellanae* isolated from northern Greece and central Italy. No other bacterial species among those tested showed an amplification product under optimized PCR conditions. The adding of 4% BLOTTO (10% skim milk powder and 0.2% NaN_3) in the PCR mixture proved essential in order to avoid interference of hazelnut extracts during the amplification. The procedure proved more effective than repetitive PCR with ERIC primer sets in diagnosing apparently healthy hazelnut trees as infected. This technique could be of great help for screening the hazelnut propagative material as well as for monitoring the wild *C. avellana* trees growing in the woods near the infected hazelnut orchards.

Introduction

Pseudomonas avellanae (Psallidas) Janse et al., is the causal agent of hazelnut (*Corylus avellana* L.) decline in northern Greece and central Italy (Psallidas, 1987; Scortichini et al., 2000a). The pathogen enters the tree through the leaf scars in autumn and, subsequently, it can systemically move to the root system of adult and young hazelnut trees (Scortichini and Lazzari, 1996). It can kill the whole plant within a period of a few months up to some years. The detection of *P. avellanae* is currently based either on traditional techniques that include pathogenicity tests which require at least 6–7 months for the completion or on repetitive-PCR using the ERIC primers and requiring the isolation and the production of pure cultures (Scortichini et al., 2000a). The latter procedure can be completed in 4–6 days but latent infection cannot be detected. The possibility of utilizing a diagnostic technique enabling a reliable and rapid screening of the propagative material, can also support the establishing of new hazelnut orchards. In fact, in central Italy as in most of the areas devoted to hazelnut cultivation, the orchards are still based on autochtonous cultivars and the sanitary assessment of the suckers is not carried out. This paper reports on the design of primers based on 16S rRNA gene sequences of *P. avellanae* and the optimization of the polymerase chain reaction (PCR) conditions for the rapid, specific and sensitive detection of *P. avellanae* in hazelnut trees and propagative plant material.

Materials and Methods

Bacterial strains, growth conditions and DNA preparation

The strains of *P. avellanae* and other bacteria used in this study are listed in Table 1. *Pseudomonas avellanae* and other pseudomonads were grown on nutrient agar with 5% of sucrose added (NSA), at 25–27°C. Xanthomonads and *Enterobacteriaceae* were cultured on glucose–yeast-extract–calcium-carbonate agar (GYCA), at 25–27°C. In addition, some unknown bacterial species, frequently obtained during the isolation of diseased hazelnut specimens were grown on NSA. For DNA preparation, pure cultures grown for 48 h on agar were suspended in sterile saline (SS; 0.85% NaCl in distilled water) and, subsequently, centrifuged three times at 10 000 × ***g***, for 2 min at 4°C. The pellet was resuspended in sterile bidistilled water and aliquots of 0.1 ml were used to start 523 broth cultures. The cultures were

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Table 1
List of bacterial strains used in this study

Strains	No. of strains	Sources and strain designation
Pseudomonas avellanae (Italy)	23	ISPaVe 012, PD 2390 = ISPaVe 013, 037, 038, 039, 040, 041,042, 056, 369, 436, 439, 689, 690, 691, 2056, 2059, ISF C1, VM 1, VM 2, VM 3, VM 4, VM 5
Pseudomonas avellanae (Greece)	17	BPIC 631^T, 632, 640, 647, 649, 659, 665, 703, 707, 708, 710, 714, 1077, 1078, 1436, Fl 3
Pseudomonas sp. pathogenic to hazelnut	35	ISPaVe 592, 593, 595, 596, 598, 599, ISF Lab 1, Lab 2, Lab 3, Lab 4, Lab 5, Lab 6, C 1, C 2, C 3, C 4, C 5, C 6, C 7, Lan 1, Lan 2, Lan 3, Lan 4, Lan 5, Lan 6, Lan 7, To 3, To 4, To 5, To 6, Cr 2, Cr 3, Cr 4, Cr 5, Cr 6
Pseudomonas syringae pv. *syringae*	22	LMG 1247_{t1} = NCPPB 281^T, 1092, 1093, 1097, 2427, 3869, PD 2618, 2631, 2633, 2634, BPIC 1509, 1556, ISF D10, D11, D12, D13, D14, D 16, D 21, D 22, D 36, D 38
Pseudomonas syringae pv. *morsprunorum*	2	NCPPB 2427, 2787
Pseudomonas syringae pv. *persicae*	1	NCPPB 2761
Pseudomonas syringae ssp. *savastanoi*	7	NCPPB 64, 639, 1006, 1506, BPIC 344, 463, 857
Pseudomonas amygdali	1	NCPPB 2607
Pseudomonas viridiflava	4	NCPPB 451, 3195, ISF B1, B 2
Pseudomonas cichorii	5	NCPPB 943, 950, 2379, 2479, 3153
Pseudomonas corrugata	8	NCPPB 2445, 2447, 2449, 2455, 2456, 2457, 2903, 3031
Xanthomonas arboricola pv. *corylina*	8	NCPPB 935, 984, 2896, 3037, 3339, PD 1896, 1897, 3657
Xanthomonas arboricola pv. *juglandis*	10	NCPPB 411, 412, 413, 414, 1659, 2927, PD 130, 157, 189, 2635
Pantoea herbicola	2	PD 127, 150
Erwinia nigrifluens	1	PD 968
Erwinia salicis	1	PD 749
Unknown from *Corylus avellana*	47	

NCPPB, National Collection of Plant Pathogenic Bacteria, York, UK; PD, Culture Collection of Plant Protection Service, Wageningen, The Netherlands; LMG, Belgian Coordinated Collections of Microorganisms, Gent, Belgium; BPIC, Benaki Phytopathological Institue Collection, Kiphissia-Athens, Greece; ISPaVe, Culture Collection of Istituto Sperimentale per la Patologia Vegetale, Roma, Italy; ISF, Culture Collection of Istituto Sperimentale per la Frutticoltura, Roma, Italy.

grown at 25–27°C for 18 h. Aliquots of 1.5 ml were then taken and centrifuged (12 000 × ***g***, 5 min, 4°C) and the cells resuspended in SS up to an optical density corresponding to $1–2 \times 10^8$ cells/ml. The suspensions were heated at 95°C for 10 min and then stored at −20°C to be used for PCR amplification.

Primer design

The partial sequence of the 16S rRNA gene (i.e. 1–1384 bases) of *P. avellanae* strain PD 2390, isolated in central Italy (EMBL bank, accession number X95745) (Janse et al., 1996) was compared with the sequence coding for the same gene of *P. syringae* pv. *syringae* van Hall, strain LMG 1247_{t1} (EMBL bank, accession number Z76668) (Moore et al., 1996) by using Oligo Primer Analysis software, Version 5.0 (National Bioscience Inc., Plymouth, MN, USA). Areas of the 16S rRNA gene exhibiting sequence variability were chosen as possible primer sequences. Primer PAV 1 (forward primer), covering positions 264–289 of the *P. avellanae* 16S rRNA gene, and PAV 22 (reverse primer), covering positions 997–1025 of the same gene, displaying similar melting points, no stable harpin and duplex structures, were chosen. The primers were synthesized by Eurogentech (Seraing, Belgium).

PCR amplification of pure cultures samples

The PCR was performed in a PTC 100 programmable thermocycler (MJ Research, Watertown, MS, USA). The PCR reaction mixture (50 μl) contained 1 × reaction buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; 0.1% Triton X-100); 1.5 mM $MgCl_2$; 100 μM of each dNTP; 12 pmol of each primer; 0.5 U *Taq* DNA polymerase (Promega, Madison, WI, USA), and 6 μl of the bacterial DNA solution. The following PCR conditions were used: initial predenaturation at 95°C for 7 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. After a final extension step of 72°C for 3 min, the mixture was stored at 4°C. After the PCR, 9 μl aliquots of the reaction mixture were resolved by electrophoresis on a 1% agarose gel in 0.5 × TBE (Tris Borate EDTA) buffer, at 4 V/cm over 2 h. DNA fragments were stained in 0.5 μg/ml ethidium bromide, visualized under a UV transilluminator and photographed with a Polaroid film type 55 (Polaroid, Cambridge, MA, USA).

PCR sensitivity

For the sensitivity assay, the procedure described by Seal et al., (1993) was followed. Suspensions of *P. avellanae* strain PD 2390 was serially diluted in sterile bidistilled water by 10-fold increments from 1×10^{11} cells/ml to 10 cells/ml. Samples of 100 μl were plated out on NSA and incubated at 25–27°C for 2–3 days. Concentrations of viable bacteria were estimated as the number of cells per ml which developed after the plating on NSA medium. Samples of 100 μl of the serial dilutions of *P. avellanae* cultures were also treated as described above for DNA preparation and aliquots of 6 μl of each dilution used for PCR amplifications following the procedures previously described.

Primer sensitivity in presence of plant extracts

In parallel, the primer sensitivity was also assessed by mixing, in 1 : 1 ratios, aliquots of *P. avellanae* suspen-

sions in sterile bidistilled water and healthy hazelnut tissues. For this purpose, twigs, roots and cortical layers of branches of hazelnut were removed and plant extracts were prepared by taking pieces of around 1 cm and by crushing them in 4 ml of sterile bidistilled water. After the mixing of the aliquots, 10 μl were added to the PCR mix for the amplification. One of the major limitations for the routine use of PCR for plant disease diagnosis is the possible presence of inhibiting compounds such as plant polyphenolics in the template (John, 1992). To resolve this possible problem, from 1 to 4% BLOTTO (10% skim milk powder and 0.2% NaN_3) (De Boer et al., 1995) was added to the PCR mix and the results were compared with the results of amplifications performed without this addition. In the presence of the plant extracts, the PCR mixture (50 μl) contained 1 × reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100); 1.5 mM $MgCl_2$; 200 μM of each dNTP; 36 pmol of each primer, 2.0 U of *Taq* DNA polymerase and from 1 to 4% BLOTTO. The PCR cycles were the same as for the amplification performed with the pure cultures as previously described.

Primer specificity

The specificity of the primers was assessed towards all strains listed in Table 1. DNA preparation, composition of the PCR mixture and PCR cycles were the same as described above for all strains. The PCR mixture, but with the DNA replaced by sterile bidistilled water, was used as negative control.

Plant material analysis

Extraction of *P. avellanae* DNA from diseased hazelnut tissues was performed by crushing small pieces (1 cm) of twigs, roots or branch tissues of hazelnut cultivars Tonda Gentile Romana and Nocchione in sterile mortars containing 4 ml of sterile bidistilled water. Ten microlitres of the extract were added to the PCR mixture in the presence of 4% BLOTTO. The PCR conditions were the same as described above. Before centrifugation, aliquots of 0.1 ml were also spread on NSA medium and incubated at 25–27°C for 3–4 days. The same procedure was adopted with apparently healthy hazelnut specimens (i.e. twigs, roots, branches) obtained from orchards growing near trees that were infected by *P. avellanae*. In total, 120 specimens were analysed (60 for each cultivar). The procedure was also applied to 20 hazelnut suckers growing at the collar level of hazelnut trees that were showing initial symptoms of decline (twig dieback).

Identification with repetitive PCR

In parallel, the samples were also analysed by applying the repetitive PCR procedure using ERIC primer sets (Scortichini et al., 2000a). Aliquots of 0.1 ml of the same suspensions as used for PCR amplification with the specific primers, were also spread, undiluted and serially 10-fold diluted, on NSA medium. The plates were incubated at 25–27°C for 3–4 days. DNA was extracted from levan-positive, oxidase-negative cultures that induced a hypersensitivity reaction in tobacco leaves and, due to their morphology, were suspected to belong to *P. avellanae*, in order to perform the repetitive PCR as described elsewhere (Scortichini et al., 2000a,b).

Results

Primer selection

Two primers were designed based on the regions of the 16S rRNA gene of *P. avellanae* strain PD 2390 which exhibited variability within the same regions of the same gene of the related species *P. syringae* pv. *syringae*. The forward primer, PAV 1, was a 26-mer with the following sequence: 5′-GGCGACGATCCGTAACTGGTCTGA-GA-3′, covering positions 264–289 of the *P. avellanae* 16S rRNA gene. The reverse primer, PAV 22, was a 29-mer with the following sequence: 5′-TTCCCGA-AGGCACTCCTCTATCTCTAAAG-3′, covering positions 997–1025. Analysis using the Oligo Primer Analysis software, version 5.0, indicated that, among the possible primers designed, this pair showed least complementarity with itself or with sequences other than the target sequence.

PCR specificity

A PCR amplification product of 762 bp that was specific for all 40 strains of *P. avellanae* isolated from central Italy and northern Greece was obtained with the PCR conditions previously described and no other bacterial species listed in the Table 1 produced an amplification product upon amplification with PAV 1 and PAV 22 primers (Fig. 1).

Primer sensitivity

As few as four cells per PCR tube, equivalent to 650 cells/ml, of *P. avellanae* strain PD 2390 could be detected using the PCR conditions described above (Fig. 2). When the aliquots of the bacterial suspensions were added to the mortars containing the plant extracts and 10 μl of the resulting mixture were directly added to the PCR tube, no band was obtained after PCR amplification. However, when BLOTTO was added to the PCR tube, the amplification band of 762 bp was detected. A concentration of 4% (v/v) BLOTTO in the PCR mix enabled the detection of a clearly resolved band (Fig. 3). This concentration was judged as optimal for the screening of the diseased and healthy hazelnut specimens.

Plant material analysis

A total of 120 samples of hazelnut cultivars Tonda Gentile Romana and Nocchione were analysed in parallel either by PCR using PAV 1 and PAV 22 as specific primers and adding 4% BLOTTO to the mix or by applying repetitive PCR using ERIC primer sets with pure cultures obtained after the isolation on NSA medium. Both methods detected the presence of the pathogen in all of the visibly infected trees of the two hazelnut cultivars (60 samples) (Table 2). When samples obtained from apparently healthy trees were analysed, the specific primers detected 50 out of 60 samples as positive, whereas the repetitive PCR detected 39 positive

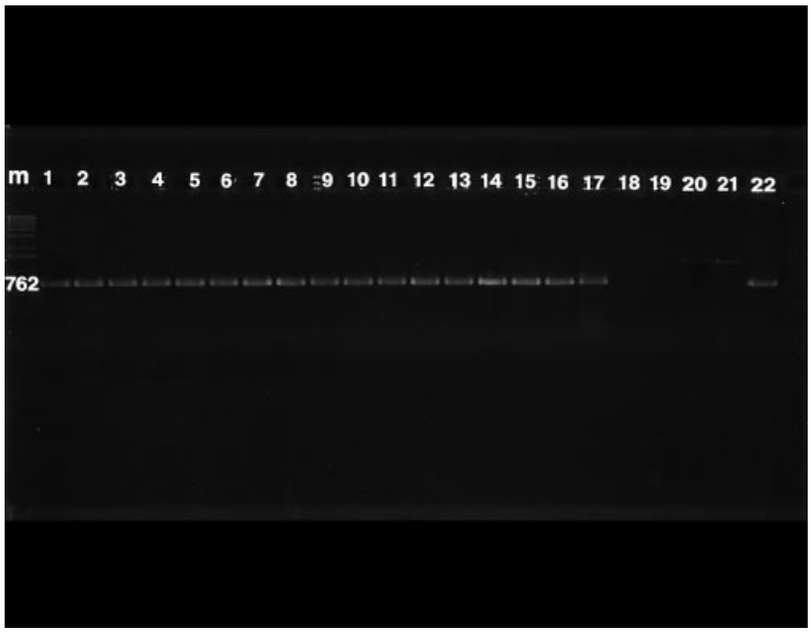

Fig. 1 Electrophoretic analysis of PCR-amplified 16S rRNA gene of different strains of *Pseudomonas avellanae* using PAV 1 and PAV 22 primers. m, molecular size marker (1 kb ladder; Gibco-BRL, Life Technologies, S. Giuliano Milanese, Italy). Lane 1, ISPaVe 037; lane 2, ISF C1; lane 3, ISF VM 1; lane 4, ISF VM 2; lane 5, ISF VM 3; lane 6, ISF VM 4; lane 7, ISPaVE 2059; lane 8, ISPaVe 369; lane 9, BPIC 703; lane 10, BPIC 640; lane 11, BPIC 665; lane 12, BPIC 631; lane 13, BPIC Fl 3; lane 14, 1077; lane 15, BPIC 1078; lane 16, BPIC 1436; lane 17, BPIC 632; lane 18, *Xanthomonas campestris* pv. *corylina* NCPPB 3339; lane 19, *Pseudomonas syringae* pv. *syringae* LMG 1247; lane 20, *P.s.* pv. *syringae* NCPPB 1097; lane 21, *P.s.* pv. *syringae* NCPPB 2427; lane 22, *P. avellanae* PD 2390

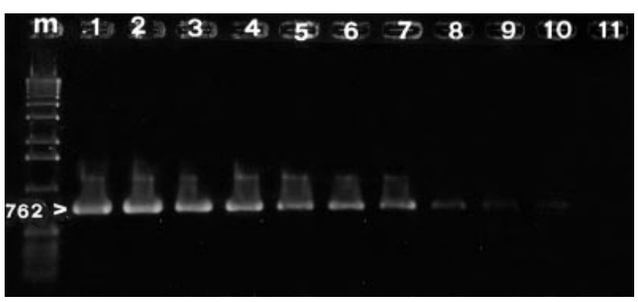

Fig. 2 Determination of the sensitivity of PCR conditions with primers PAV 1 and PAV 22 using *Pseudomonas avellanae* PD 2390. m, molecular size marker (1 kb ladder; Gibco-BRL). Lanes 1–10, dilutions of *P. avellanae* cells ranging from 1×10^{11} to 6.5×10^{2} colony-forming units/ml

out of 60 (Fig. 4). The 11 trees that were found positive with PCR amplification with primers PAV 1 and PAV 22 and negative with repetitive PCR showed symptoms of decline some months after the test. All 10 apparently healthy trees that were tested negative after the screening with the specific primers, did not show any visible symptoms of decline after 1 year from the test. The PCR with PAV 1 and PAV 22 primers detected the presence of *P. avellanae* in 11 of the 20 suckers, whereas the repetitive PCR only detected it in six.

Discussion

A rapid and sensitive procedure has been developed for the specific detection of *P. avellanae* in hazelnut samples. A discrete DNA fragment of 762 base pairs related to the 16S rRNA gene sequence of this pathogen, was specifically amplified from targeted DNA templates extracted either from pure cultures or from visibly infected as well as from apparently healthy hazelnut trees. The protocol described here allows specific diagnosis within 6 h of receiving the samples. In contrast, repetitive PCR requires at least 4–6 days (Scortichini et al., 2000a). Primers PAV 1 and PAV 22, which were chosen after comparison of the partial sequence of the 16S rRNA gene of *P. avellanae* strain PD 2390, isolated

Fig. 3 Gel electrophoresis of PCR products obtained from pure cultures of *Pseudomonas avellanae* with primers PAV 1 and PAV 22 adding BLOTTO (10% skim milk powder plus 0.2% NaN_3) to PCR mixture. m, molecular size marker (1 kb ladder, Gibco BRL). Lanes 1–3, PCR mixture tube with 4–1% BLOTTO added; lanes 4–6, PCR mixture tube without addition of BLOTTO

Table 2
Detection of *Pseudomonas avellanae* in hazelnut samples

	Tonda Gentile Romana						Nocchione					
	Twig		Branch		Root		Twig		Branch		Root	
	D	AH	D	AH	D	AH	D	AH	D	AH	D	AH
PCR using PAV 1 and PAV 22	10[a]/10[b]	10/10	10/10	8/10[c]	10/10	8/10[c]	10/10	9/10[c]	10/10	8/10[c]	10/10	7/10[c]
Repetitive PCR using ERIC primers	10/10	8/10[d]	10/10	7/10[d]	10/10	6/10[d]	10/10	7/10[d]	10/10	6/10[d]	10/10	5/10[d]

D, samples showing visible symptom of decline; AH, samples taken from apparently healthy trees. [a]Positive test result; [b]number of samples tested; [c]all 10 trees that tested negative were still healthy 1 year after the test; [d]at least one tree for each batch was found to be visibly infected within 1 year after the test.

Fig. 4 Gel electrophoresis of PCR products from DNA extracted from apparently healthy hazelnut trees (lanes 1–15) and visibly infected trees (lanes 16–18), using PAV 1 and PAV 22 primers. BLOTTO (4%), was added to the PCR mixture tube before amplification. m, molecular size marker (1 kb ladder; Gibco BRL). In lanes 1, 3, 4, 9, 10 and 11 the 762 bp band is visible indicating that the samples are positive for the presence of the pathogen

in central Italy, with the sequence of the same gene of *P.s.* pv. *syringae* type-strain LMG 1247_{t1}, allowed such a specific amplification. These primers were also efficient in recognizing all 40 *P. avellanae* strains isolated either in central Italy or in northern Greece, thus confirming the genetic relation between these two populations (Janse et al., 1996; Scortichini et al., 1998). The templates from Xanthomonads and *Enterobacteriaceae* as well as from unknown species frequently found during the isolations from hazelnut specimens did not produce any discrete band upon amplification. The primers also discriminated *P. avellanae* from the fluorescent pseudomonads pathogenic to *C. avellana* in other regions of Italy, thus confirming that such populations are poorly related to *P. avellanae* (Scortichini et al., 2000b). An important step of this procedure is the adding of BLOTTO at 4% (De Boer et al., 1995) to the PCR mix before the amplification, as the PCR inhibitors are also present in hazelnut tissues. Indeed, the procedure only detected the pathogen in mortars in which non-culturable *P. avellanae* cells were probably present when BLOTTO was added to the mix. The screening of plant specimens also allowed the detection of the pathogen in apparently healthy hazelnut trees and suckers. In such cases, the number of samples in which *P. avellanae* was detected by PCR using PAV 1 and PAV 22 primers was higher than the number of positive samples using repetitive PCR and ERIC primer sets. This is particularly significant for the detection of latently infected hazelnut trees and it could be of great help in the assessment of propagative material as well as for testing wild *C. avellana* trees growing near to infected hazelnut orchards. In fact, there could be a risk for the spreading of decline from the orchards to the forests (Scortichini et al., 2000c).

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